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SHORT COMMUNICATION

Role of the Conserved Dipeptide Gly75 and Cys76 on HIV-1 Vpr Function

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Vpr is one of the accessory proteins encoded by the HIV-1 genome. Several interesting features associated with Vpr include incorporation into virus particles, ability to oligomerize, localization in the nucleus, and positive effect on virus production and replication. In order to understand the structure–function relationship of Vpr, we have analyzed the role of the Gly75 and Cys76 (GC) residues which are highly conserved in HIV-1 Vpr and in Vpr and Vpx of HIV-2/SIV. We have generated several substitution mutants involving this dipeptide and have evaluated for expression, stability, nuclear localization, and virion incorporation of Vpr. Our data demonstrate that the GC residues are not essential for virion incorporation and nuclear localization of Vpr. Serine substitution for Cys, however, restricted the localization of Vpr in the cytoplasm without affecting the Gag-directed incorporation of Vpr into virus-like particles. Interestingly, the cysteine-substituted mutants showed altered stability in comparison to the wild type, and substitution mutants for glycine showed minimal effect on stability. These results indicate that the glycine and cysteine do not play a role in nuclear localization or virion incorporation properties of Vpr and further suggest that these two functions of Vpr may not be interdependent.

HIV-1 Vpr is one of the accessory genes that is relatively conserved among HIV-1 and -2/SIV (*22, 29*). Vpr is a late protein synthesized in infected cells and the generation of mRNA corresponding to Vpr is dependent on Rev function (*1, 10, 14*). Vpr is immunogenic and a considerable proportion of infected individuals (30–40%) have antibodies to this protein (*15*). The biological studies carried out with macrophage-tropic viruses showed that Vpr is essential for the productive infection of macrophages (*2, 3, 39*). Interestingly, the replicative ability of the Vpr-defective virus was not significantly different in comparison to wild type in established T cell lines (*2, 6, 11, 30*). Further, a recent report showed that HIV-1 Vpr participates in the transport of viral DNA to the nucleus in nondividing cells (*17*). The requirement of Vpr for macrophage infection has been demonstrated for HIV-2 as well (*16*).

HIV-1 Vpr is a protein of 96 amino acids in length except for that of HIV-1_{SF2} which has 97 amino acids (*29*). Genetic organization of HIV-1 Vpr is similar to Vpr and Vpx of HIV-2/SIV. Tristem and co-workers have reported the presence of several conserved residues along the entire coding sequence (*37*). Biochemical and genetic studies carried out with Vpr revealed several interesting properties: (i) association with virus particles (*7, 20, 40*), (ii) localization in the nucleus (*24, 41*), (iii) ability to oligomerize (*4, 31, 42*), (iv) interaction with a cellular protein (*41*), (v) induction of differentiation in a human tumor cell line (*21*) and arrest of cells at G2 stage of the cell cycle (*25, 32*), (vi) participation in the transport of viral DNA to the nucleus (*17*), and (vii) exertion of influence on viral production and replication (*2, 22*). Mutational analysis carried out by several groups revealed important residues for Vpr function (*26, 27, 31, 41, 42*). It was reported that arginine residues (amino acid positions 32, 62, 73, 88, and 95) are not essential for Vpr incorporation into virus particles and Vpr containing an alteration in the cysteine residue was not detectable in cells upon transfection of proviral DNA (*32*). There is also conflicting data regarding the role of C-terminal residues in nuclear localization (*24, 41*) and virion incorporation of Vpr (*31, 38*). Recently, work carried out in our laboratory on Vpr identified an amino-terminal helical domain that is essential for the incorporation of Vpr into virus particles (*26, 27*). To learn more about the structure–function relationship of Vpr, we have targeted the highly conserved glycine–cysteine (GC) (amino acid positions 75 and 76) residues as these residues may have an important role in the function of Vpr.

Alignment of the amino acid sequence of HIV-1 Vpr derived from macrophage-tropic molecular clone 89.6

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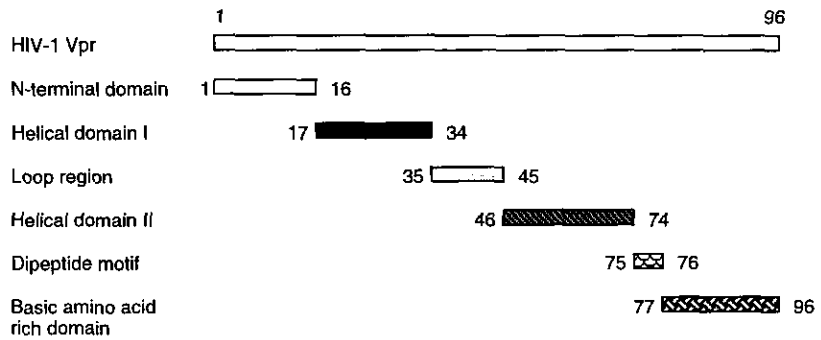

FIG. 1. Schematic diagram of the putative domains of HIV-1 Vpr. The numbers indicate the positions of the predicted amino acid residues of Vpr.

with the sequences of Vpr and Vpx of HIV-2/SIV revealed several conserved residues as reported previously (*37*). These include residues 2, 13, 18, 29, 30, 33, 35, 36, 51, 65, 71, 75, and 76. The conservation of these residues suggests that these may participate in the functions directed by this accessory gene product. Secondary structural analysis using Chou–Fasman algorithm previously reported an acidic α-helical domain at the amino-terminus (residues 17–34) of Vpr. However, the hydrophobic moment calculations using GCG package suggest an additional α-helical structure in the middle region (residues 46–74) of Vpr (Fig. 1). The two putative helices are connected by a loop structure (residues 35–45). The C-terminus of Vpr has a basic amino acid-rich region (residues 77–96) downstream of the conserved dipeptide (GC; residues 75 and 76) (Fig. 1).

Since glycine and cysteine are conserved in HIV-1 Vpr and in Vpr and Vpx of HIV-2/SIV and cysteine has been shown to play a major role in the stability of proteins and also in protein–protein interactions (*5, 9, 12, 28, 35, 36, 43*), we have targeted the Gly and Cys for mutagenesis to evaluate the role of this motif on the expression, stability, subcellular localization, and virion incorporation function of HIV-1 Vpr. The amino acid substitution mutants for glycine and cysteine were constructed by overlap PCR method. Based on the contradictory biological data with regard to the stability of Vpr available for cysteine-substituted mutants from our laboratory (*26*) and that of Paxton and co-workers (*31*), we have considered generating several substitution mutants involving polar, nonpolar, and basic amino acid residues in place of cysteine. Similarly, the conserved glycine was also changed to alanine and arginine. The details of the mutants are shown in Fig. 2A.

We employed vaccinia virus–T7 RNA polymerase-based expression system (vTF7-3) (*13*) to study the effect of mutations in the dipeptide motif. vTF7-3-infected HeLa cells were transfected with wild-type or mutant Vpr expression plasmids by the lipofectin method. Cells Vprwere labeled for 2 hr, lysed, immunoprecipitated with anti-Vpr antiserum, and analyzed on SDS–12% PAGE. As expected, the cells transfected with Vpr expression plasmid showed a protein of 14 kDa in size, while the mock transfected and pCDVprΔ-transfected cells failed to show the corresponding protein (Fig. 2B). Glycine- and cysteine-substituted mutant Vpr expressed efficiently upon transfection into HeLa cells. Expression of Vpr directed by the arginine-substituted Vpr mutant (VprC76-R) showed an altered migration in comparison to wild type and other cysteine-substituted mutants.

Cysteine residue has been implicated in the stability of proteins. This has prompted us to analyze the stability of mutant Vpr in cells. Plasmids encoding wild-type or mutant Vpr were transfected into vTF7-3-infected HeLa cells and pulse labeled for 30 min. After different chase periods, cell lysates were immunoprecipitated with anti-Vpr antibody and subjected to SDS–12% PAGE. Wild-type Vpr is stable in cells for the chase period of 10 hr used in this study. However, the cysteine-substituted mutants showed altered stability in comparison to the wild type (Fig. 3A). In general, all the cysteine-substitution mutants showed reduced stability at the end of the 10-hr chase. Similar studies with glycine-substitution mutants showed minimal effect on the stability of Vpr (Fig. 3B). These results suggest that cysteine residue may contribute to the stability of Vpr.

To address the role of dipeptide motif GC on the incorporation of Vpr into virus-like particles directed by HIV-1 Gag, we transfected the vTF7-3-infected HeLa cells with a Gag expression vector (pCDGag) in combination with wild-type or mutant Vpr expression plasmids. Immunoprecipitation of Vpr and Gag was performed both in cell lysates and in culture media using anti-Vpr antiserum and HIV-1 antiserum after 5 hr of continuous labeling (Fig. 4). When cell lysates were examined, Vpr was found in cells transfected with wild-type and all mutant Vpr plasmids, with or without gag cotransfection (data not shown). When the supernatant was tested, either by direct examination of supernatant (data not shown) or after size-exclusion centrifugation to select for particle-associated protein, expression of Vpr alone did not result in the export of Vpr into the cell culture media (Fig. 4A). Expression of Gag resulted in a 55-kDa product in the

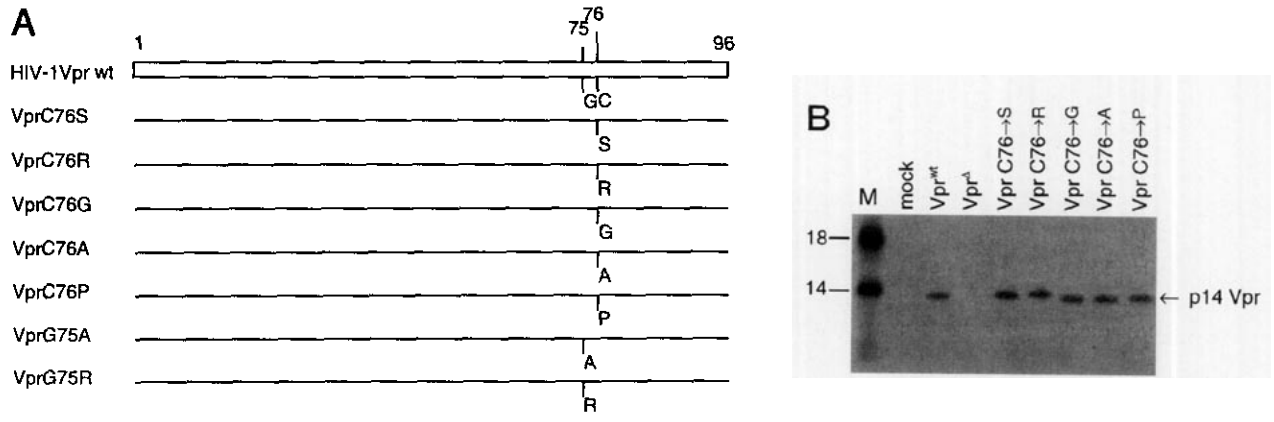

FIG. 2. Construction and expression of mutant Vpr. (A) Plasmids containing the mutant Vpr were generated by overlap polymerase chain reaction (PCR) at the indicated codons (*18*). PCR-amplified mutant *vpr* gene fragments using proviral DNA as template (*8*) were digested with *Hin*dIII and *Xho*I and ligated to pCDNA3 vector to produce mutant Vpr expression plasmids (*34*). The PCR primer sequences will be made available upon request. The mutant Vpr plasmids were verified by DNA sequence analysis. (B) Recombinant vaccinia virus (vTF7-3)-infected HeLa cells were transfected with wild-type and mutant Vpr expression plasmids. Transfected cells were labeled with ^{35}S protein labeling mix for 2 hr and the cell-associated Vpr proteins were immunoprecipitated with anti-Vpr antiserum as described previously (*26, 27*). Immunoprecipitates were analyzed by SDS–12% PAGE. The designations of the Vpr plasmids are indicated at the top.

cell culture supernatant and, as expected, coexpression of Vpr and Gag resulted in the export of Vpr into the culture medium in association with virus-like particles (Fig. 4). Glycine- (Fig. 4B) and cysteine-substitution mutants (Fig. 4A) also showed incorporation of mutant Vpr into virus-like particles as efficiently as wild type. These results clearly indicate that glycine and cysteine are not essential for the Vpr incorporation into virus particles.

In an effort to evaluate the role of the dipeptide motif in the subcellular localization of Vpr, we examined the glycine- and cysteine-substituted mutants for intracellular localization by immunofluorescence assay. The wild-type Vpr, as expected, localizes in the nucleus (Figs. 5A and 5B). Both glycine- (data not shown) and cysteine-substituted Vpr mutants also localized in the nucleus except serine substituted for cysteine Vpr mutant (Figs. 5C–5G). This Vpr mutant, Vpr C76S, localized primarily in the cytoplasm (Fig. 5E) and exhibited the phenotype of virion incorporation like that of wild type. These results suggest that nuclear localization may not be an essential feature of Vpr for its incorporation into virus particles.

In our effort to determine the structure–function relationship of Vpr, we have targeted previously the amino-terminal acidic domain (helical domain I) identified by Chou–Fasman algorithm (Fig. 1). The substitution mutation analysis involving residues in this α-helical domain showed that this is an essential domain for several functions of Vpr including stability, nuclear localization, and virion incorporation (*26, 27*). In this study, we have analyzed the role of conserved Gly75 and Cys76 which are present in HIV-1 Vpr and in Vpr and Vpx of HIV-2/SIV on HIV-1 Vpr function. Vpr is an auxiliary protein encoded by

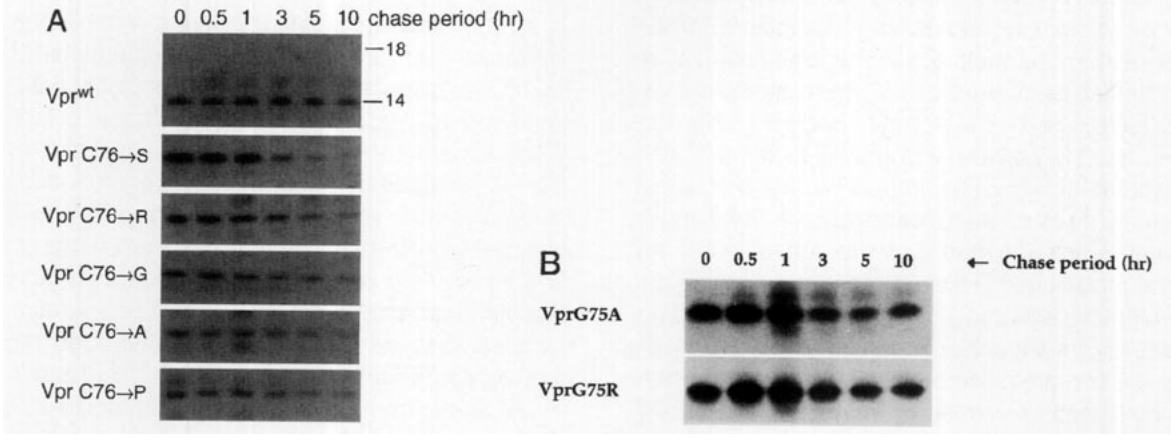

FIG. 3. Stability of cysteine and glycine Vpr mutants. (A) Cysteine 76 Vpr mutants. Wild-type and mutant Vpr expression plasmids were transfected into the vTF7-3-infected HeLa cells. Transfected cells were pulse labeled for 30 min with 200 μCi of ^{35}S protein labeling mix and chased for different periods. Cells were then lysed and the cell-associated Vpr protein was immunoprecipitated with anti-Vpr antiserum and analyzed by SDS–12% PAGE. Chase periods are indicated at the top. (B) Glycine 75 Vpr mutants.

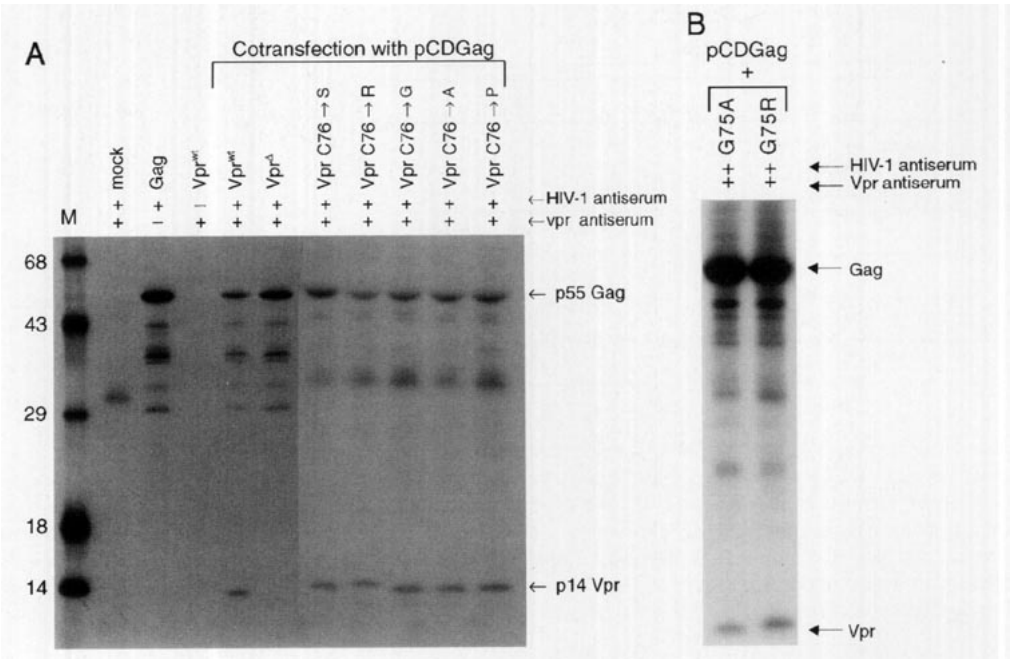

FIG. 4. Incorporation of Vpr into virus-like particles directed by Gag. Cotransfection of pCDGag with wild-type or mutant Vpr expression plasmids was carried out using vTF7-3-infected HeLa cells as described previously (*26, 27*). Transfected cells were labeled with ^{35}S protein labeling mix for 5 hr, the culture medium was cleared by centrifugation and concentrated using Centricon 30 concentrators, and virus-like particles were resuspended with RIPA buffer. Immunoprecipitation was carried out using anti-HIV and anti-Vpr antisera and analyzed by SDS–12% PAGE. (A) Cysteine and (B) glycine Vpr mutants. The electrophoretic positions of Gag and Vpr are shown at the right and molecular mass markers are shown at the left in kilodaltons.

HIV genome and a number of biological and biochemical characteristics associated with this protein have been identified (*29, 34*). Currently there is limited information available regarding the essential domains of Vpr underlying these properties. Further, the interrelationship between these characteristics in the overall function of Vpr is not clear. Importantly, the cysteine residue has been implicated in the interactions of proteins (*5, 9, 12, 19, 28, 35, 36, 43*). It has been shown that disulfide bonds between cysteines are crucial for the proper folding and stability of many proteins (*9, 38*). Further, it has also been suggested that disulfide bonds are responsible for the oligomerization of proteins (*28*). The presence of an unique cysteine residue in Vpr may participate in intramolecular interactions and it is also likely that intermolecular interactions between Vpr may also be mediated by the cysteine residue. To verify this, we introduced different amino acids at the cysteine residue. Substitution of the Cys residue with Ala, Pro, Gly, Arg, and Ser did not alter the properties of Vpr with respect to virion incorporation function. Strikingly, the mutant Vpr showed an altered stability in comparison to wild-type Vpr. Studies regarding the subcellular localization of mutant Vpr indicate that both glycine- and cysteine-substituted mutants showed nuclear localization of Vpr similar to wild type except the serine substitution for cysteine. This mutant resulted in Vpr that failed to localize in the nucleus. Also, cysteine-substitution mutants exhibited oligomerization similar to wild-type Vpr (data not shown) which indicates that cysteine may not be involved in the oligomerization of Vpr. This conclusion is also supported by a recent report which implicates the amino-terminus of Vpr to be involved in the oligomerization function (*42*). The results reported here with respect to substitution at the cysteine residue are contrastingly different from those of a previous report (*31*). Paxton and co-workers have mutated the cysteine residue in a modified Vpr (epitope-tagged Vpr) to alanine in the context of proviral DNA (*31*). Upon transfection of proviral DNA containing mutant Vpr into cells, Vpr was not detectable by immunoprecipitation using antiserum specific to epitope tag in comparison to wild type. This observation led them to conclude that the cysteine-substituted mutant Vpr degrades quickly in cells. In this experiment these authors were unable to evaluate the role of cysteine-substitution mutant for its ability to incorporate into virus particles. The basis of the discrepancy between the data reported here and those of Paxton and co-workers is not clear (*31*). The differences such as epitope addition to Vpr (*31*) and T7 polymerase-based expression system used here may contribute to the contrasting experimental data observed.

The studies presented here indicate that the cysteine residue is an essential component for the stability of Vpr as Vpr is highly stable in cells (*26*). The stability of proteins can result from intra- and intermolecular interaction of proteins involving the disulfide bonds between the

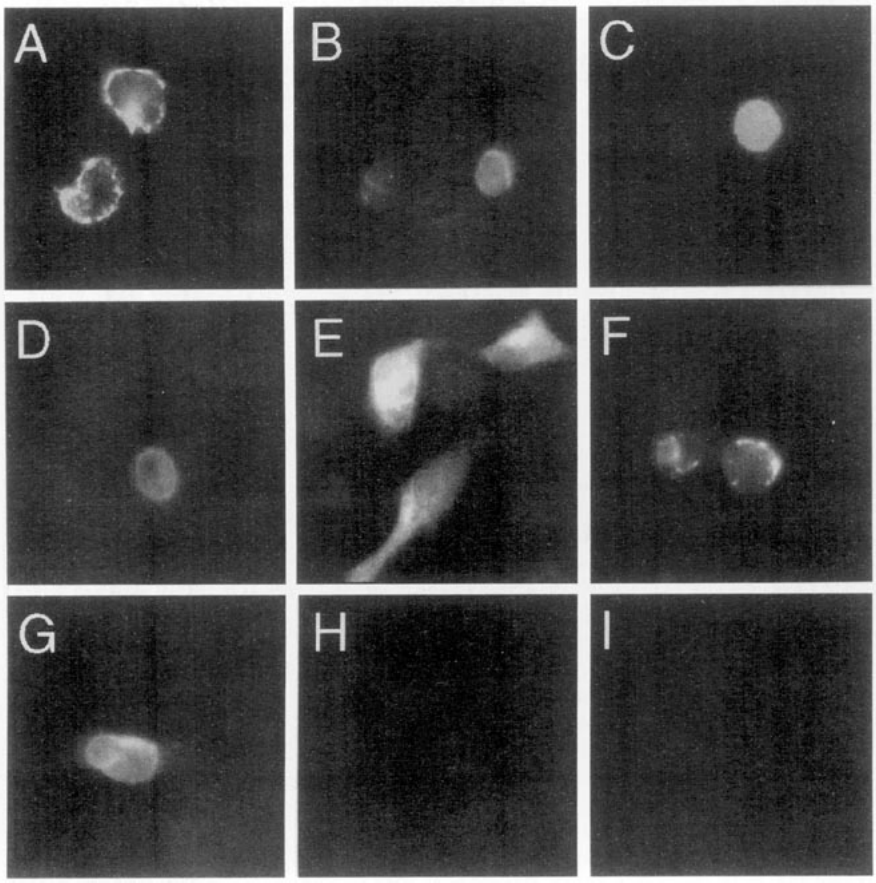

FIG. 5. Subcellular localization of Vpr. HeLa cells were maintained in DMEM containing 10% fetal bovine serum and seeded on to poly-L-lysine-coated glass coverslips at the density of 1×10^6 cells per dish (35 mm). Twenty-four hours later they were infected with vTF7-3 and transfected as described previously (*26, 27*). Sixteen to 24 hr after transfection the cells were washed with PBS and fixed with methanol at room temperature for 30 min. The cells were then washed with PBS and incubated for 90 min with primary antiserum (1:50). After washing with PBS the coverslips were incubated for 90 min with FITC-conjugated affinity-purified F(ab)'2 fragment of goat anti-rabbit IgG (ICN Biochemicals, CA) diluted 1:100 in PBS and washed six times with PBS. Coverslips were then counterstained for 5 min with Evans Blue (0.02% in PBS; Sigma, St. Louis, MO) and again washed prior to being mounted on glass slides using a fade-resistant mounting medium (Citiflour, UK). All incubations were carried out at 37° in a humidification chamber. The primary antibody was a polyclonal rabbit antiserum raised against a 96-amino-acid Vpr synthetic peptide (*27*) and preimmune serum from the same animal was used in parallel as a control. (A) and (B) wild-type Vpr; (C) C76A; (D) C76P; (E) C76S; (F) C76R; (G) C76G; (H) ΔVpr; (I) wild-type Vpr stained with preimmune serum. Photos are at magnification ×695.

Cys residues. It is possible that oligomerization of Vpr involving the cysteine residue through disulfide bond formation may contribute to the stability of Vpr. However, this interpretation is unlikely as Vpr containing substitution of amino acids for glycine and cysteine still exhibits oligomerization properties similar to wild-type Vpr (data not shown). The underlying mechanism(s) by which cysteine promotes Vpr stability is not clear. The results reported here and our previous studies (*26, 27*) support a model in which Vpr has distinct features modulated by different functional domains. The amino-terminal helical domain has been reported to be essential for stability, virion incorporation, and nuclear localization of Vpr (*26, 27*, unpublished data). Studies with cysteine mutants showed that the conserved cysteine may contribute to the stability of Vpr. Further, the results indicate that nuclear localization and virion incorporation features of Vpr are not interdependent. Additional studies with mutants involving other domains of Vpr should be useful in understanding the interrelationship between oligomerization, nuclear localization, virion incorporation, and the biological effect of Vpr.

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